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Feasibility study of an active wound dressing based on hollow fiber membranes in a porcine wound model

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ABSTRACT

Investigation: A novel active wound dressing (AWD) concept based on a microporous hollow fiber membrane network was investigated in an animal model. It provides a local solution-perfused environment for regenerative cell nutrition, wound irrigation, debris removal, electrolyte balancing, pH regulation, and topical antibiosis. The device is capable of supplying soluble factors, as tested experimentally for the recombinant human growth and differentiation factor-5 (rhGDF-5).

Methods: Following *in vitro* studies for rhGDF-5 using primary human keratinocytes and dermal fibroblasts, we employed a porcine partial thickness wound model with five distinct wounds on each back of $n = 8$ pigs. Four wound groups were perfused differently over 9 days and compared with a negative control wound without perfusion: (1) 1% trehalose solution, pH 5.5; (2) rhGDF-5 (150 ng/ml) in 1% trehalose solution, pH 5.5; (3) nutrition solution; and (4) rhGDF-5 (150 ng/ml) in nutrition solution with 1% trehalose, pH 5.5.

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Abbreviations: AWD, active wound dressing; BMP, bone morphogenetic protein(s); FCS, fetal calf serum; HDF, human dermal fibroblast(s); HFM, hollow fiber membrane(s); HK, human keratinocyte(s); NC, negative control; NUT, NUTRIFLEX solution setup; NUT+G, rhGDF-5 (150 ng/ml) in NUTRIFLEX nutrition solution with trehalose 1% solution setup; PC, positive control (healthy pig skin); rhGDF-5, recombinant human growth and differentiation factor-5; TRE+G, rhGDF-5 (150 ng/ml) in trehalose 1% solution setup; VAS, visual analog scale.

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Results: Promoted wound healing was observed within group 1 and more pronounced within group 2. Groups 3 and 4, with nutrition solution, showed significant adverse effects on wound healing ($p < 0.05$).
Conclusions: The investigated AWD concept appears to be an interesting therapeutic tool to study further wound healing support. Additionally, topical application of rhGDF-5 could be promising.

1. Introduction

Burn patients and patients suffering from chronic wounds have significantly lowered chances to effectively recapitulate lost skin function through natural healing by re-epithelialization. Burn victims start to lose natural vasculature at the time of injury. The microvascular system for delivery and removal of molecular and cellular wound healing building blocks is already impaired in partial thickness wounds (second-degree burn). A loss of capillaries in damaged skin results in accumulation of fluids and a shift of signal molecules, such as growth factors, where they are needed most.

As a consequence, cell supply is not optimal in the early phase of wound healing, electrolyte de-arrangements occur and toxins accumulate. This leads to pH fluctuations and generally to an unphysiological wound environment. To improve wound healing, we believe it could be interesting to address the damaged natural capillary system temporarily with an artificial replacement, as part of the wound dressing.

Conventional wound dressings, including gauze, polymer films, foams, and gels are incapable of providing a temporary and localized control of soluble factors within the wound bed and addressing the above issues. Silver sulfadiazine dressings were found to be consistently associated with poorer healing outcomes with biosynthetic on silicon-coated dressings, whereas hydrogel-treated burns showed improved healing outcomes over those treated with usual care [1]. Although systemic reviews of relevant literature were frequently conducted, it still appears impossible to draw firm and confident conclusions about the effectiveness of specific conventional dressings such dressings play a passive role during wound healing and predominantly in addressing the wound closure aspect. Therefore, providing a controlled wound irrigation cannot be realized. To overcome this situation, active wound dressings (AWD) were designed to provide convective mass transfer within the wound bed [2].

When designing an AWD system, it is crucial that a moist wound environment, prevention of microbial activity, and removal of exudates or toxins are considered. In this regard, a variety of wound dressings are available, however, not all meet the specific requirements of an ideal wound healing system to consider every aspect within the wound-healing cascade [3].

Wound dressings, specifically with a constant wound irrigation and liquid perfusion aspect, appear promising. Davis et al. [4] chose a simultaneous irrigation and negative pressure wound therapy (NPWT) approach in a full-thickness excisional wound inoculated with *Pseudomonas aeruginosa*. The authors found that NPWT with simultaneous irrigation further reduced the bioburden over control-treated wounds, whereby flow rate did not affect these outcomes [4].

The AWD, that we tested, is based on microporous hollow fiber membrane (HFM) capillaries and is intended to mimic an artificial capillary bed that can deliver any soluble factor to the wound site, while enabling continuous irrigation with solutions under a constant perfusion with adjustable negative pressure. By using an artificial capillary network, a decentralized mass exchange can be provided. Accordingly, a disposable artificial vascular system for temporary use should promote initial tissue engineering in the wound of the patient; and also provide a milieu in which regenerative cells sprayed into the wound would be supported as in a cell culture incubator. The device concept, with illustrations, was introduced elsewhere [5]. To temporarily replace the lack of sufficient vascularization in a wound, like chronic venous ulcers or burns, we used perfused microporous polyethersulfone hollow fiber ultrafiltration membranes (MicroPES TF10, Membrana, Wuppertal, Germany).

Thus, major aspects of healing strategies may be combined in one AWD concept; *i.e.*, controllable irrigation for keeping the wound moist, controllable perfusion for debris removal, antibiosis, nutrition, pH regulation, electrolyte balancing, detoxification, and topical factor supply.

Growth and differentiation factor activities, particularly, were described in every phase of skin wound regeneration [6–9]. During regeneration, a shift of endogenous factor concentrations was observed, often with pathophysiologically undesirable alterations, due to negative feedback mechanisms [10–15]. Therefore, compensation by topical delivery of external factors has been taken into account and various approaches were described [16–18]. However, optimal delivery systems and strategies for continuous local factor application are still being debated [19,20]. The AWD concept, involving continuous perfusion, potentially addresses the challenges of a short therapeutic window, irregular application characteristics, and undesirable shifts in doses of regenerative factors.

The investigated device has the potential of supplying factors in physiologically relevant dosages without generating application peaks. Moreover, similar to systemic kidney dialysis, the AWD may create a local solution-perfused environment for regenerative cell nutrition and it enables wound irrigation for milieu regulation and topical antibiosis.

In the present study, feasibility and safety of this concept were investigated *in vivo* using a porcine model. Using a HFM patch prototype (Fig. 1) and different irrigation solutions based on combinations of trehalose and NUTRIFLEX[1] nutrition

[1] Words in capital letters indicate trademarks throughout the text.

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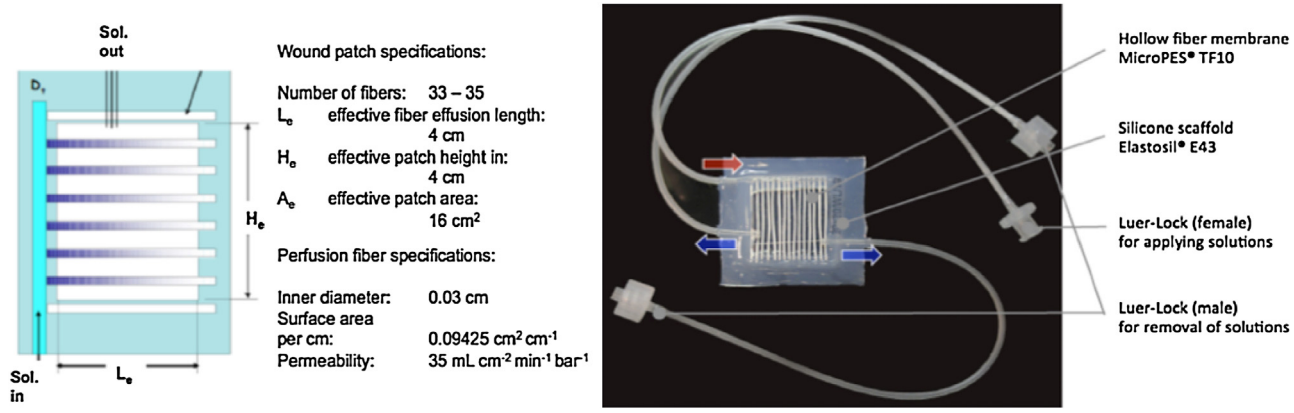

Fig. 1 – Prototype animal wound patch. On the left side a technical scheme is depicted with specifications and demonstrating unidirectional perfusion. On the right side the construction of the animal wound patch is exemplarily shown (not to scale).

solution, with or without the factor rhGDF-5 as experimental candidate for applicability of regenerative mediators, the impact on porcine wound recovery was assessed.

In several tissues [21], GDF-5 has been shown to promote cell proliferation, differentiation and/or tissue formation. Further, GDF-5 is naturally occurring in human skin [21–23] and was described to exhibit angiogenic properties [24,25]. The factor also is thought to promote neuronal induction [26,27]. Initial data suggested its regenerative potential for skin tissue [23,28]. Accordingly, this factor is of interest for acute and chronic wounds and reflects a relevant model protein for growth factor delivery by the AWD.

Our hypothesis is that acute skin wound healing is supported by the presented AWD concept. To test this, first, we conducted a preliminary *in vitro* activity study of rhGDF-5 employing primary human keratinocytes (HK) and primary human dermal fibroblasts (HDF) cultures. Afterwards, the impact of the AWD and rhGDF-5 delivery on wound regeneration was analyzed in an acute surgical porcine wound model. We employed a standardized surgical split-thickness excision wound pattern, followed by application of active wound-dressing prototypes and various irrigation solutions. This model excludes an application of negative pressure. As parameters, we chose perfusion characteristics, capillary plugging, encapsulation of capillaries in the wound bed, wound patches' integrity, obvious allergic reactions, and systemic effects. For assessment of the wound healing progress, we chose the macroscopic parameter (i) visual gross appearance (macroscopic outcome), (ii) visual re-epithelialization, (iii) skin color score, (iv) wound distortion score, and (v) avoidance of wound infection during application, and the histologic parameter of H&E stained cellular layer appearance and thickness.

2. Materials and methods

For details on basic *in vitro* characterization of rhGDF-5 delivery to skin cell cultures, see supplemental materials.

2.1. Experimental irrigation solutions

The rhGDF-5 factor was purchased from BIOPHARM GmbH, Heidelberg, Germany where it was recombinantly produced in *Escherichia coli* K-12 W3110 as described elsewhere [29]. The 3.1 mg/ml rhGDF-5 was diluted in 10 mM HCl to obtain 1 mg/ml stock solution of rhGDF-5. One gram T9531 D-(+)-trehalose dihydrate (Sigma–Aldrich, Taufkirchen, Germany) was diluted in a 100 ml 5 mM Na-acetate (pH 5) and filtrated to obtain 1% trehalose solution. NUTRIFLEX Peri infusion solution (B. Braun Melsungen AG, Melsungen, Germany) was purchased ready-to-use.

2.2. Wound groups

The investigated wound groups 1–5 are characterized by the device and irrigation solution applied and abbreviated as follows:

Group 1 (TRE): trehalose 1% solution in *aqua ad injectionem* and 5 mM sodium acetate; pH 5.5.
Group 2 (TRE+G): rhGDF-5 (150 ng/ml) in 1% trehalose solution in aqua ad injectionem, 5 mM sodium acetate; pH 5.5.
Group 3 (NUT): NUTRIFLEX nutrition solution.
Group 4 (NUT+G): rhGDF-5 (150 ng/ml) in 1% trehalose dissolved in NUTRIFLEX nutrition solution and 5 mM sodium acetate; pH 5.5.
Group 5 (NC): dry negative control wound without perfusion; wound covered with V.A.C. foil (Kinetic Concepts, Inc., San Antonio, TX) and surrounded by a ring of silicone tubing.

The positive control (PC): healthy skin area on the back of each pig.

2.3. Prototype device

The experimental patch consists of a standardized HFM MICROPES TF 10 (Membrana GmbH, Wuppertal, Germany) with 50 mm × 50 mm in size (see a schematic model in Fig. 1).

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The HFM capillaries were cut at the two opposite ends. On the left side, the lower half of 100 mm long VMQ silicone tubing, 25 mm × 30 mm in diameter (Deutsch & Neumann, Berlin, Germany), was cut and wrapped around the HFM to incorporate the open capillaries in the lumen of the tubing. At the end of the upper intact half of the tubing, a female Luer Lock connector was affixed and sealed airtight. On the right side, the opposite ends of the HFM capillaries were completely sealed with silicone rubber ELASTOSIL E43 (Wacker, Munich, Germany). The entire HFM was embedded in a square-shaped medical grade silicone scaffold (width of approx. 20 mm). Accordingly, the animal wound patch was open to the wound site only for unidirectional perfusion of irrigation solutions (see technical scheme on the left of Fig. 1). On the top and bottom sides, the silicone scaffold was opened with VMQ silicone tubing; approx. 50 mm in length (Deutsch & Neumann, Berlin, Germany) was placed atop the HFM for removal of used irrigation solutions. At the opposite ends, both tubes were sealed airtight with a Luer Lock connector. All tubes were incorporated in the silicone scaffold. In order to protect the filigreed capillaries from the environment and to reduce spillage and evaporation of irrigation solutions, the animal wound patch was covered with a thin layer made of ELASTOSIL E43 silicone rubber. The silicone scaffold further served to fix the patches on the back of the pigs *via* surgical sutures.

2.4. Animal study

The approval for the experimental protocol for the study was obtained by the Federal German authorities for animal research; LAGeSo (Berlin, Germany). The principles for laboratory animal care followed the guidelines of the European and German Society of Laboratory Animal Sciences.

2.5. Animals, housing and general anesthesia

Eight healthy German Landrace cross breed pigs, four male and four female with an average body weight of 50 ± 5 kg, and age of 3–4 months were housed pairwise for 10–14 days of habituation. General housing conditions were applied as described previously [30]. General anesthesia was also described elsewhere [31].

2.6. Porcine wound model

The pigs were placed in a prone position in the operating theater. Using a sterile battery dermatome HUMECA D80 (Humeca B.V., Enschede, The Netherlands), five wounds were created on the backs of the pigs under aseptic conditions. The wounds were equal in size (50 mm × 50 mm), depth (1.2 mm) and rectangular orientation to the spine. The wounds were superficial dermal excisional wounds, simulating second-degree burns, *i.e.*, epidermis and upper third of the dermis were removed. Although an equal wound pattern was set in each pig, alignment of wound treatment differed among the pigs to randomize the study. After creation of the wounds, blood was allowed to coagulate to create a blood film on the surface of wounds. After hemostasis was achieved, the coagulated blood was removed. To prevent further coagulation on the wound surfaces, they were covered with phosphate buffered saline (PBS; PAA Laboratories, Cöelbe, Germany) soaked gauze. Fixation of wound patches was realized *via* surgical sutures. Further, the HFM was flushed several times with PBS to further avoid blood coagulation during fixation. Initial flushing of experimental irrigation solutions followed, resulting in a reservoir left in the wounds. A custom-made protective coat, consisting of two fabric sheets, covered the wounds and was sutured on both flanks of the pig. Both sheets were connected by hook and loop tape in order to enable continuous access to the wounds and for wound patch observation.

After surgery, two pigs were held individually for 9 days for investigation purposes. Analgesia was maintained for 6 days post surgery with fentanyl patches (DURAGESIC 75 μg/h, Johnson & Johnson, Neuss, Germany). The experimental irrigation solutions were changed twice a day—once in the morning between the hours of 10 and 11 a.m., and once in the late afternoon between the hours of 5 and 6 p.m. Prior to the application of 10 ml of fresh solution for each wound, the used solution was suctioned off *via* syringes through the tubes lying atop the HFM. Afterwards, syringes filled with 10 ml of fresh and sterile irrigation solution, for each respective wound group, were perfused through the HFM *via* the connected tubing. The wound patches remained fixed throughout the study—no dressing changes were made. All pigs were sacrificed on day 9 with an overdose of sodium thiopental (1 kg) followed by potassium chloride (60 mmol).

2.7. Blood sampling

Blood sampling at the baseline (day 0) and at the end of the experiment (day 9) was conducted for each pig to obtain an overall clinical picture and to evaluate potential systemic effects during the study. Parameters of a complete blood count, including leukocytes, erythrocytes, hemoglobin, hematocrit, and the platelets were determined by an external laboratory for veterinary medical diagnostics (SYNLAB, Berlin, Germany). Blood glucose, lactate and pH were analyzed using a radiometer analyzer ABL 700 (Radiometer, Copenhagen, Denmark).

2.8. Assessment of wound healing progress

Wound healing progress was determined macroscopically and microscopically at day 9 after surgery. Also, standardized photo documentation and biopsy sampling of the lesions were conducted. Prior to this, all lesions were gently cleansed with saline soaked gauze to remove adhered exudates and used wound solutions.

Macroscopic assessment was descriptive and based on visual gross appearance. In addition, we focused on the individual attributes of re-epithelialization expressed in % of re-epithelialized wound surface (area [%]), as well as on a skin color and wound distortion score. For microscopic assessment, we focused on H&E stainings with descriptive analysis of overall epidermal, granulation tissue, and dermal appearance at 10× microscopic photographs. Particularly, we chose histomorphometric analysis of the thickness of cellular layers

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as the most important characteristic for the histologic outcome.

2.9. Macroscopic assessment

All wounds were digitally photographed (NIKON COOLPIX S2500, Nikon, Tokyo, Japan) with approximately the same distance, angle, and under similar light conditions.

The observer's overall impression of the wound pictures was subjectively graded from poor outcome (below NC group outcome), moderate outcome (comparable to NC group as reference), improved outcome (promoted outcome compared to NC group), and excellent outcome (comparable to healthy skin). Thus, descriptive analysis focused on the criteria of homogeneity in wound appearance, contrast of wound margins to surrounding skin, and visual healthy skin restoration.

2.10. Re-epithelialization

Further, macroscopic re-epithelialization was determined while drawing a rectangular grid, consisting of 10×10 squares, over the wound pictures using a customized macro in ImageJ 1.45 s [32,33]. Each square represents 1% of a 50 mm × 50 mm wound.

Therefore, each square with visual healthy skin appearance was considered the re-epithelialized area (see *e.g.*, Fig. 2e) in the center of the wound for reference. Accordingly, macroscopic re-epithelialization is expressed in area [%]. Each wound group was analyzed this way for $n = 8$ and the corresponding area [%] were expressed as mean ± SD in Fig. 3.

2.11. Microbial smear tests

Immediately after removal of wound patches microbial smear tests ("D2" sterile cotton swab; Heinz Herenz Medizinalbedarf GmbH, Hamburg, Germany) were conducted on each individual wound to investigate the antibiosis aspect of the wound perfusion system. The tests were sent to a specialized lab for analyses (Charité Campus Virchow-Klinikum, Berlin, Germany).

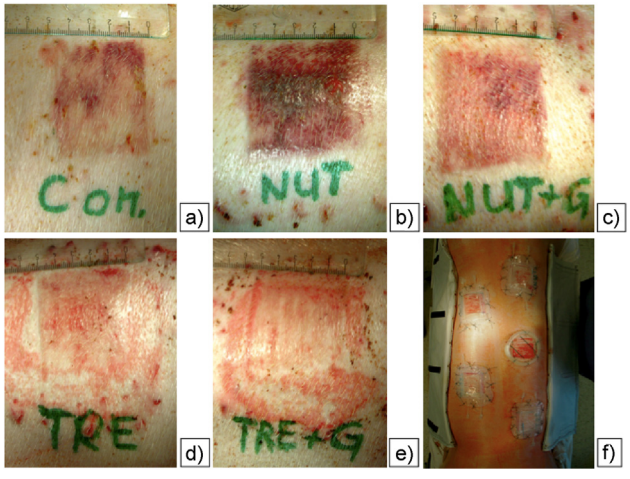

Fig. 2 – Display of the experimental groups; one pig on final day 9: (a) NC; (b) NUT; (c) NUT+G; (d) TRE; (e) TRE+G; (f) overview with opened swine coat; NC group is shown in the center on the right. In (a) abbreviation "Con." represents NC group, *i.e.*, negative control without perfusion.

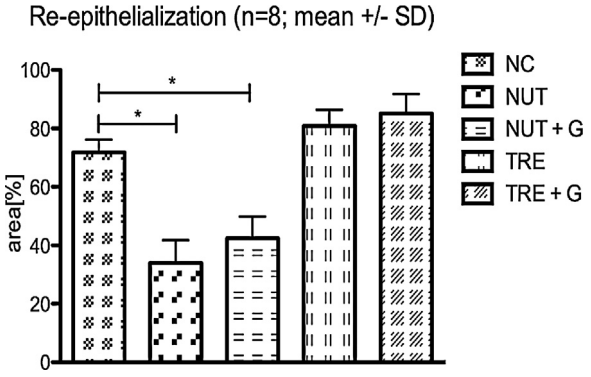

Fig. 3 – Visual re-epithelialization in area [%] for $n = 8$ pigs is expressed as bar chart with mean ± SD; asterisk indicates $p < 0.05$.

2.12. Microscopic assessment/H&E staining

Biopsies were taken from the center of all lesions and the healthy tissue from the back of each pig. Afterwards, the full-thickness excisions were transferred into 4% paraformaldehyde. Sections were embedded with paraffin, and later trimmed automatically in a tissue processor (Shandon Hypercenter XP, Thermo Shandon, Pittsburgh, PA). Next, the biopsies were cut on a microtome (Microm HM355s, Microm International GmbH, Walldorf, Germany) into 3 μm thick sections and stained with hematoxylin and eosin (H&E) for routine histology analyses following a standard protocol.

2.13. Histomorphometric analysis

All H&E stained sections were captured using a CCD-camera (QIMAGING, Surrey, Canada) at 10× magnification (AXIOVERT microscope 200, Zeiss, Göttingen, Germany) and digitally transferred into imaging software (JPEG, QCAPTURE PRO 6.0 Software, QIMAGING, Surrey, Canada) with a resolution of 2048 × 1536 pixel. Analysis of cellular layer appearance was descriptive. We focused on the epidermis, including rete ridges, granulation tissue, and its organization along with the remaining dermis appearance. Thereby, the NC and PC group served as controls to evaluate the appearances as improved (similarities to healthy skin); normal (comparable to NC group); slightly abnormal (appearance below that of NC group), and diffuse (appearance far below NC group as reference). Further, the data was recorded by computer-assisted morphometric analysis using ImageJ 1.45s [32,33] for the histological sections. For thickness measurements, in each H&E section, three perpendicular axes were measured for distance in randomly selected regions of interest (ROIs) from the epidermal layer and the underlying granulation tissue ($n = 3$ for each section). The distance was recorded in pixel and

transferred to mm. Hereby, a scale bar Leitz 1/100 mm (Leitz, Wetzlar, Germany) was used for calibration of each section analyzed. Images were analyzed in a blind fashion and by random sampling to obtain unbiased results. Mean values and standard deviation (SD) were considered for data evaluation of thickness measurements.

2.14. Statistics

One-way analysis of variance (ANOVA) was performed followed by Dunnett's multiple comparison tests for analyzing the data obtained. For all evaluations, $p < 0.05$ constituted statistical significance. GraphPad PRISM 5.0 (GraphPad Software, Inc., La Jolla, CA) was used for statistics and for illustration. Unless otherwise indicated, all values are expressed as mean ± SD.

3. Results

All pigs ($n = 8$) underwent eventless surgery and experimental phase. Blood analysis on day 9 of investigation, in comparison to day 1, confirmed that no pig showed signs of systemic infections or any comorbidity that could have affected the wound outcome during the study (data not shown).

3.1. The rhGDF-5 factor in vitro characterization

Naturally occurring GDF-5 and its corresponding receptors are expressed in porcine skin. The respective lane for the GDF-5 factor and its receptors BMPRII, BMPRIA, ACTRIIA, BMPRIB, ACTRIIB in the Agarose gel of Fig. S1 (see supplemental materials) reflect the presence of its native transcripts in porcine skin. The GAPDH control was also positive. During HFM capillary binding studies, the optimal delivery and activity of rhGDF-5 were observed when dissolved in 1% trehalose aqueous solution containing 5 mM sodium acetate of pH 5 (see Table S2 and Fig. S2 in the supplemental materials). A subsequent dose-finding study using HK and HDF *in vitro* indicated that 150 ng/ml rhGDF-5 can be used for the animal study (see Figs. S3 and S4 in the supplemental materials).

3.2. Active HFM wound dressing

HFM perfusion characteristics in the *in vivo* situation were normal. A reservoir of irrigation solution was maintained under the wound patch to address moist wound treatment as well. No blood clotting and plugging could be observed on the HFM capillaries or in the tubing. The solution perfusion and removal was possible up to day 9 of the investigation. Regenerative skin cells did not overgrow the HFM capillaries. In this respect, microscopical review of HFM, after detachment, was negative in each case. Therefore, flushing the HFM with fresh experimental irrigation solutions twice a day was effective to avoid capillary clotting by any wound material. Identification of HFM fabrication concerns for *in vivo* wound application was negative. No abnormal wound surface or allergic reaction due to HFM application could be observed visually. Importantly, the HFM remained intact in each case. No ruptures of the filigreed capillaries could be observed. The surgical sutures kept the wound patches adequately in place.

3.3. Macroscopic outcome

A representative outcome of the wound groups is exemplified for one pig in Fig. 2. Visual assessment of clinically relevant re-epithelialization in area [%] is depicted in Fig. 3. The macroscopic scoring of all investigated pigs for skin color and wound distortion is depicted in Fig. 4.

In the following paragraphs, the macroscopic outcome is described for each wound group.

Wounds of the NC group were not fully resurfaced in all cases. Re-epithelialized areas were observed predominantly on the wound margins. The center of the NC wounds still showed dark red to light red areas indicating non-healed wound surfaces (see Fig. 2a as an example). Therefore, moderate homogeneity of wound surface with contrasting wound margins to surrounding skin and moderate healthy skin restoration characterized the NC group macroscopic outcome.

All wounds treated with the NUTRIFLEX nutrition solution (NUT group) showed impaired healing, as dark red areas, bloody debris, and strongly contrasting wound edges were observed in all cases (see Fig. 2b as an example). All wound surfaces showed no areas of homogeneous appearance. All wounds were barely or not visually re-epithelialized and considered a poor macroscopic outcome.

Likewise, the rhGDF-5 in 1% trehalose solution and the NUTRIFLEX solution setups (NUT+G group) showed impaired healing. However, macroscopic impression of the NUT+G wounds differed to a greater extent among the animals, and in a few cases an improved outcome could be observed compared to NUT wounds (see Fig. 2c as an example). Nevertheless, the overall picture of the NUT+G group is characterized by no homogeneity of wound surfaces, strongly contrasting wounds to the surrounding skin, and barely or non-epithelialized areas. Thus, the NUT+G group was considered for poor macroscopic outcome as well.

As compared to the NC wounds, the 1% trehalose setups (TRE group) showed positive results; however, its macroscopic

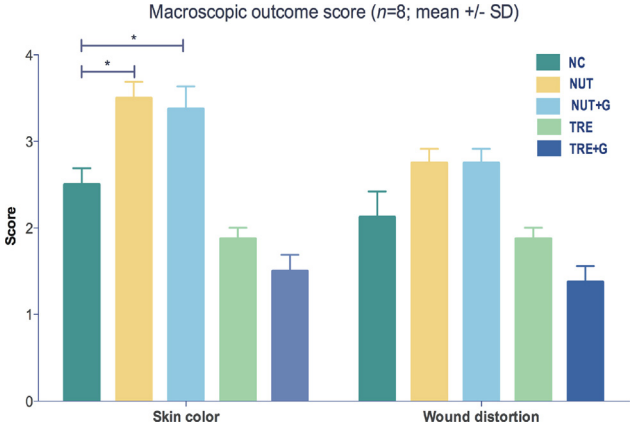

Fig. 4 – Macroscopic outcome score for skin color and wound distortion ($n = 8$) is expressed as bar chart with mean ± SD; asterisk indicates $p < 0.05$.

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impression differed among the animals. The wound edges still remained contrasted. Smaller dark red to light red areas were visible and skin distortion still occurred (see Fig. 2d as an example). Wound surfaces; however, presented a higher degree of homogeneity as compared to NC group and could be considered for an improved macroscopic outcome. Slightly bloody debris was only observed in a few cases.

By contrast, the rhGDF-5 in 1% trehalose solution setups (TRE+G group) exhibited the highest visible rate in reconstitution of new epithelium (see Fig. 2e as an example). Although slight differences still were visible compared to healthy skin, on the final day 9 most wounds showed 75–90% re-pigmented areas with homogenous surface appearance. In most cases wound edges were harmonized in color with the surrounding healthy skin (see Fig. 2e as an example). Neither bloody debris nor any signs of hypergranulation nor otherwise deteriorated wound healing was observed. Therefore, the TRE+G group was considered for an improved macroscopic outcome as well.

3.4. *Re-epithelialization/skin color/wound distortion*

The re-epithelialization rates, expressed in area [%], reached highest values for the TRE and the TRE+G group, indicating best progress in macroscopic wound healing. By contrast, the NUT+G, and more pronounced the NUT group showed delayed re-epithelialization, reflected by lower values when compared to the NC group (see Fig. 3). Although, significance for the TRE and TRE+G group over the NC group could be not determined by the present feasibility study on HFM wound patches, a positive trend in wound healing progress was detectable. This was further supported by skin color and wound distortion score (see Fig. 4), which reached the lowest values indicating least contrasting wound surfaces with least bloody debris, but a high degree of wound surface homogeneity. Specifically, the TRE and the TRE+G group scored below a 2 for skin color, indicating predominantly less reddened areas, whereas a score above 3, for the NUT and NUT+G group, reflected predominant red and dark red areas.

3.5. *H&E staining*

Fig. 5 illustrates representative microscopic photographs (10×) for the histological findings. A fully differentiated and well-stratified epidermis, including a functional *stratum corneum*, was only observed in the TRE+G group (see Fig. 5f as an example). The beginning of rete ridges restoration was detected in all cases ($n = 8$). Granulation tissue was present in all cases and appeared organized and homogenous. The dermal layer exhibited normal appearance. Therefore, for the TRE+G group, an improved histological appearance could be detected. Compared to that the TRE group without rhGDF-5, the delivery exhibited visibly thicker epidermal layer and thickened granulation tissue (see Fig. 5e as an example). In all H&E stained sections, spots of disorganized areas could be observed for both the epidermal and granulation tissue layer. Restoration of rete ridges was evident only in three cases. The dermal layer of the TRE group exhibited normal appearance. Therefore, the TRE group was considered as a normal histological appearance. The epidermis of the NC group was not fully differentiated (see Fig. 5b as an example)

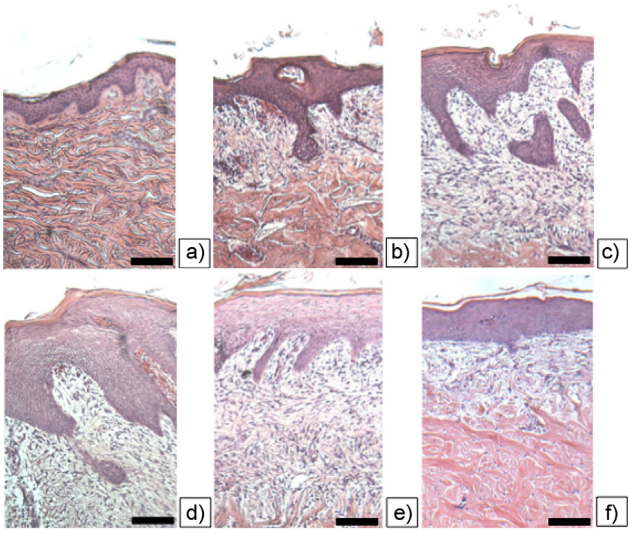

Fig. 5 – H&E stained sections of the experimental groups (10× magnification); one pig on final day 9; (a) PC; (b) NC; (c) NUT; (d) NUT+G; (e) TRE; (f) TRE+G. Scale bar = 300 μm each.

and remained thickened when compared to healthy skin (see Fig. 5a as an example). The epidermis, granulation tissue, and the reconstituted dermis all still showed disorganized tissue sections in the NC group (see Fig. 5b as an example). No restoration of rete ridges was observed. Both NUTRIFLEX treated wound setups, *i.e.*, NUT and NUT+G group, led to an excessively thickened epidermis and granulation tissue on day 9 of the investigation. Further, in both groups, cellular whirls occurred and loss of cellular stratification was evident (see Fig. 5c and d as an example) and no rete ridges were observed. Accordingly, both NUTRIFLEX treated wound groups were considered for diffuse histological appearance.

3.6. *Histomorphometric analysis*

Figs. 6 and 7 show histomorphometric measurements for epidermal thickness and for granulation tissue thickness, respectively, on final day 9. For all pigs ($n = 8$) the thickness of healthy epidermis (PC group) was 0.066 mm mean ± 0.023 mm SD, and served as a reference for evaluating the treatment outcome with respect to epidermal reconstitution (see Fig. 6).

The NC group exhibited a more than doubled mean epidermal thickness when compared to healthy skin. The mean thickness of granulation tissue of the NC group was 0.357 ± 0.198 mm and served as reference for evaluating granulation tissue thickness (see NC in Fig. 6). During H&E analysis, only in two sections of the NUT group could an encapsulation be observed of the capillaries in wound tissue (data not shown).

The TRE+G group showed the closest results to healthy skin tissue with a mean epidermal thickness of 0.113 ± 0.064 mm. The granulation tissue was close to the reference values of the NC group.

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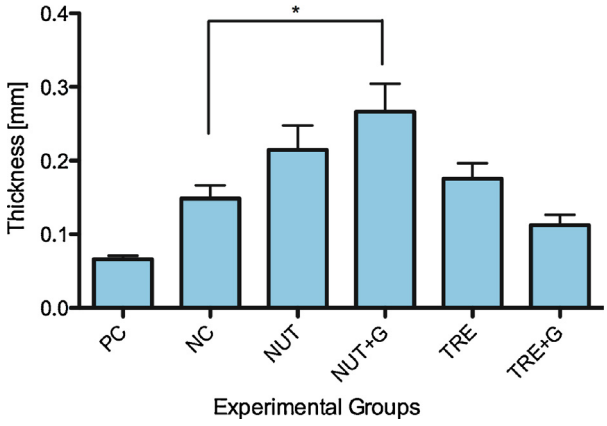

Fig. 6 – Histomorphometric analysis of epidermal thickness in mm of H&E stained sections of the experimental groups for $n = 8$ pigs expressed by mean ± SD on final day 9; asterisk indicates $p < 0.05$.

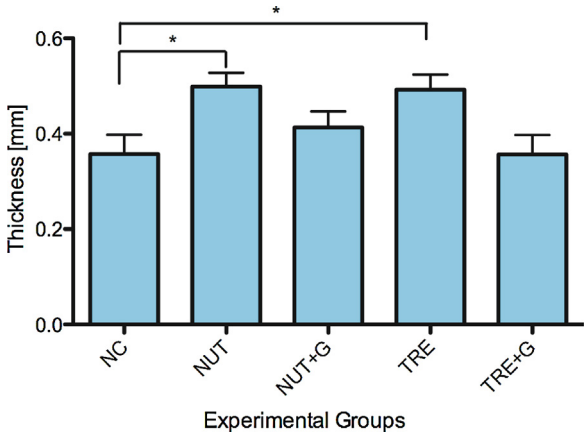

Fig. 7 – Histomorphometric analysis of granulation tissue thickness in mm of H&E stained sections of the experimental groups for $n = 8$ pigs expressed by mean ± SD on final day 9; asterisk indicates $p < 0.05$.

3.7. *Antibiosis*

Microbial colonization of investigated wound surfaces was moderate on average, as confirmed by wound smear cultures (data not shown).

4. Discussion

The complex wound healing process in skin includes various types of cells and mediators such as, cytokines, growth factors, proteases, and extracellular matrix components act in concert to restore skin's integrity [2].

The loss of capillaries in acute burns, but also in chronic wounds, results in accumulation of many negative factors contributing to delayed re-epithelialization. This leads to an unphysiological wound environment. Active wound dressings can contribute to keeping a wound moist, which is vital to promoting skin regeneration without scarring [34]. Further features are described in Section 1.

We described an *in vivo* study for a novel AWD based on HFM (introduced elsewhere [5]), while testing different experimental irrigation solutions optionally containing a skin regenerative factor candidate rhGDF-5 for topical supply.

For testing the effects of wound irrigation and topical factor supply, we employed a porcine wound model. Several porcine wound models were proposed [35,36]; however, we focused on a model described by Singer et al. [18] and included standardized excisional dermatome wounds [37] to obtain directly comparable wound sites. Unlike heterogeneous human burns or chronic wounds, dermatome wounds, in such a surgical model, are quite consistent in size, depth, and location and therefore effects of experimental manipulations on wound healing progress can be accomplished easier. Furthermore, in such porcine wounds, the subjective clinical assessment of wound healing can be readily compared with corresponding histological analysis of sampled biopsies.

Our porcine wound model proved to be adequate for the described feasibility study while testing an AWD *in vivo*. Adequate distances between the wounds of the back ensured that each wound remained individual and distinct from each other, thus avoiding cross-contamination.

By conducting studies with $n = 8$ pigs of comparable age and weight, we have not seen any remarkable differences among the wounds of the NC group. In this regard, differences are related to gross visual appearance, re-epithelialized areas, skin color, wound surface homogeneity, and H&E findings. This implies significant differences among the animals within the NC group could be not detected. Therefore, we conclude that intra-individual variations of natural wound healing were negligible; thus, the evaluated wound healing progress on day 9 can be related to the applied experimental irrigation solutions. Thus, using five wounds in eight animals compensated for variation in response to the investigated treatment options.

Exogenous growth factors were previously introduced for use in skin wound healing strategies [16–18,38]. However, a therapeutic application of proteinaceous growth factors can be challenging. Mature proteins may exhibit poor solubility in physiological solutions as wound healing is complex and endogenous growth factor levels are shifted by the impaired milieu and a need for investigating novel and promising regenerative factors remains. The growth and differentiation factor 5 (GDF-5) was tested initially in a wound-healing model *in vitro* and *in vivo* [23,28].

We studied using a supply of topical rhGDF-5 as an experimental test perfusion *via* the prototype of an AWD based on perfusable medical-grade HFM (for illustrations see [5] and Fig. 1). In advance, we had to enable full HFM passage of rhGDF-5. Further, solubility had to be addressed. Some commonly used additives for increasing the solubility of proteins or for avoidance of adsorption, such as albumin amino acids or sugars, may trigger adsorption to the materials used for factor transfer or within the wound dressings itself. A pH-dependent solubility profile of mature rhGDF-5 showed

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precipitation in liquid solutions with a pH above 4.25 and was almost insolubility within the range of pH 5–9 (data not shown). Thus, we recommend a pH range of 5–6, when rhGDF-5 is applied topically. Accordingly, we employed a stable aqueous rhGDF-5 formulation for pre-clinical applications based on a 3.1 mg/ml of rhGDF-5 diluted in 10 mM HCl to obtain 1 mg/ml of stock solution, which was equivalent to the pH following a strategy of using solutions that reduced adsorption. While adding the carbohydrate trehalose—of interest for medical grade perfusion solutions—the rhGDF-5 showed a passage of HFM capillaries without measurable loss by adsorption and without lost of its bioactivity (see Table S2 and Fig S2. in the supplemental materials). This was a prerequisite for the animal testing to enable bioactive rhGDF-5 delivery to the wound sides.

The results of cell culture assays on human epidermal and dermal skin cell monolayers as described in the supplemental materials showed a migration and proliferation stimulating potential of rhGDF-5 *in vitro*.

Using a custom-made cotton coat, applied over the wound dressings, prevented the pigs from damaging the dressings while scratching their backs. The excisional split-thickness wound model appeared adequate for testing our hypothesis, since none of the NC wounds fully re-epithelialized within nine experimental days. Further, the mean epidermal thickness measurement was more than doubled, in comparison to the healthy skin. This means that native wound healing was still in progress; normally, the epidermis and granulation tissues decreases in thickness toward the end of healing phase. As a result, on day 9 as a fix point in time for our assessment, we could compare the wounds for progress in healing with a non-spontaneously healed wound as a negative control. Differences could be observed among the studied wound groups in regard to its macroscopic and histological outcome.

Based on the macroscopic results, the wound groups were ranked in the following order from the first rank (best outcome) to last rank (poorest outcome): (1) TRE+G group, (2) TRE group, (3) NC group, (4) NUT group, and (5) NUT+G group.

The results of the H&E stained sections showed the same ranking for outcome. Specifically, a multilayered, well-differentiated epidermis, including a functional *stratum corneum* and initiated reconstitution of the rete ridges, was only evident in the TRE+G group within 9 days post surgery. By contrast, the epidermis of the untreated NC group was not fully differentiated and signs for reconstitution of rete ridges were rare. The 10× magnification for H&E analysis was considered sufficient to provide for an overview on skin cellular layers and potential impacts by the different treatment options.

A complete healing did not take place in the TRE+G group, which could not be expected within the first 9 days post surgery. The same applies to the NC group. However, as stated above our wound model was adequate to assess the wound healing progress in the early phase after injury. A positive trend for promoted wound healing was evident in all cases of the TRE+G group ($n = 8$) and to a lesser extent for the TRE group. An epidermal restoration was visible in large areas above 75% wound surface within the TRE+G group. The wound surface appeared homogenized and the least distortion occurred. The mean epidermal thickness measurement of the TRE+G group was close to the data obtained for the PC group as a healthy reference. This may reflect the beneficial effects of the 150 ng/ml rhGDF-5 in the 1% trehalose solution on re-establishing the epidermal barrier. Moreover, solely in the TRE+G group, the granulation tissue exhibited a low mean diameter accompanied by a high degree of organization. In this regard, the granulation tissue of the 1% trehalose treated wounds (TRE group) exhibited increased mean diameters when compared to the untreated NC group and the TRE+G group. In addition, the granulation tissue was found more disorganized in the TRE group. Accordingly, the beneficial effects recited for the TRE+G group could not be observed within the TRE group, suggesting again a positive effect of rhGDF-5 alone. While only in a few cases, the overall macroscopic impression of the TRE group did not differ vitally when compared to the TRE+G group—the histologic findings revealed more differences. The mean epidermal thickness measurement of the TRE group was on average one third higher when compared to the TRE+G group. Hence, an impact of rhGDF-5 on promoting re-epithelialization and organizing the granulation tissue may be concluded, while 1% trehalose solution alone showed beneficial effects under regular irrigation conditions.

Unexpectedly, the NUT group, as well as the NUT+G group, showed the worst results throughout the study. Although, the NUTRIFLEX solution was assumed to be supportive, since providing for a sufficient nutrition environment in theory, only adverse effects on wound healing were observed. All wounds perfused with NUTRIFLEX solution were the least re-epithelialized and the most contrasting to surrounding healthy skin. The H&E staining of biopsy sections revealed that both the epidermis and the granulation tissue were completely disorganized.

No differentiated epidermal layers could be observed and a cornified layer was absent in most cases. The epidermis was diffuse and thickened excessively. Particularly, cellular whirls that invaded the granulation tissue could be observed in some cases. This resulted in higher values for mean epidermal thickness during morphometric analysis of both NUTRIFLEX treated wound groups (NUT group, NUT+G group). Moreover, the granulation tissue appeared excessively thickened. It seems that rhGDF-5 could not significantly reverse a negative impact of the NUTRIFLEX solution.

The negative controls rank as intermediate between the outcome for the two 1% trehalose groups and the two NUTRIFLEX groups. Round nodules of collagen, within the granulation tissue, indicated porcine scarring and could be observed only in the case of the NUT and NUT+G groups. Therefore, we considered these solutions unsuitable the AWD concept.

The described AWD is based on perfused HFM and is intended to act as a temporary artificial capillary bed with a set of ''arterial'' inlet mass exchange hollow fibers that can deliver soluble factors to the wound site and a set of ''venous'' fluid and exudate-carrying mass exchange fibers that also serve to remove toxins from the wound bed. In previous studies, ethylene oxide sterilization was found to be adequate for inert

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sterilization of the wound patches, and not affecting the capillaries' performance (data not shown).

The HFM perfusion characteristics during *in vivo* study were not changed. No blood clotting or plugging could be observed on the HFM capillaries or in the tubing. The complete solution change, twice a day with 10 ml of fresh solution, was adequate to prevent the system from any plugging, which may otherwise hamper perfusion characteristics. In only two wounds, some encapsulation of the artificial capillaries was observed within the wound tissue. In all other cases, the surrounding cells did not attach to the artificial capillary surface. This is an important requirement of an AWD for avoiding ingrowth of capillaries into the wound bed, which may worsen the outcome during removal of the dressings.

In addition, we could not identify HFM material concerns. These medical grade fibers did not lead to visible allergic reaction locally or systemically. Also, the mechanical burden, for the fixed patches, was tolerated well and the HFM remained intact in each case.

Chronic wounds also represent a potential target for antimicrobial active wound dressings, as they are highly susceptible to become colonized. Therefore, a local and continuous supply of antibiotics by an active wound dressing may be desirable. The microbial smear test results suggest that the tested prototype wound dressing provided an adequate antibiosis.

Our work shows some limitations. Invasive analysis was not conducted during experimental phase to avoid disruption of the fragile neo-epidermis, impairments during wound healing, and to prevent disruptions of the filigree capillaries in the animal wound patches. Thus, we could not observe the wound healing process on a daily basis, since the devices were fixed with surgical sutures and changing the dressings intermittently was not advisable. Therefore, assessment of time-to-heal poses an interesting parameter in future studies. We focused on a nine-day observation period and found this time frame suitable for the proof-of-concept study. In animal models, only longer-term investigations could give valid information on a potential reduction of scar development. In addition, the animal model was not suitable to provide a constant perfusion situation, as the pigs could not show ''compliance''.

Using sutures, instead of the anticipated future application of skin glue such as Dermabond Advanced (Ethicon, Inc., Somerville, NJ) in a clinical setting, the patches did not seal completely resulting in some non-measurable loss of perfusate. This made a simultaneous negative pressure application impossible.

In conclusion, we have described an AWD concept that can be used as a wound irrigation perfusion, and delivery system for a growth factor supply. Local application of antimicrobials and/or enhancers of wound healing appear possible, while irrigation with 1% trehalose buffer solution alone promoted wound healing as well.

Our experiments support the project hypothesis, showing a positive effect of a perfusate-irrigated active wound dressing device. This device provides a wet wound healing environment that may include an optional local antibiosis. Using a porcine model, no negative systemic effects could be observed and an easy applicability was demonstrated. A positive trend, in a comparison to the controls, was constantly seen in the TRE and more pronounced in the TRE+G groups. The experimental factor rhGDF-5 applied in such a prototype AWD appears to be capable of promoting proliferative and migratory processes *in vitro* and *in vivo*. The AWD offers a novel approach for maintaining moist wound beds in burn and chronic wound victims.

Further studies are of interest for finding dosages and an evaluation of safety and efficacy in the human wound situation since—despite some similarity between human and porcine epidermis and dermis—animal models lack ease of application under constant perfusion and clinical complexity.

Author contributions

Jörn Plettig and Jörg Gerlach designed and coordinated the work, developed methods and prototypes, analyzed data and wrote the manuscript. Christa Johnen, Kirsten Bräutigam, Katrin Zeilinger, Eva C. Wönne, and Fanny Knöspel developed methods, analyzed data and provided discussions. Frank Schubert designed and refined the HFM wound patch prototypes. Frank Plöger analyzed data and provided discussions for the rhGDF-5 *in vitro* and *in vivo* experiments. Juliane K. Unger developed methods and coordinated the work for the animal study. Reinhard Bornemann developed methods and prototypes, and provided discussions.

Conflict of interest

Jörg C. Gerlach licensed IP to StemCell Systems GmbH, Berlin, Germany, which provided prototypes for the study and has a financial interest in the technology. Stem Cell Systems GmbH, with Reinhard Bornemann as CEO, employed Frank Schubert, as well as temporarily Jörn Plettig and Christa M. Johnen. Biopharm GmbH, Heidelberg, Germany, holds various patents on the human growth and differentiation factor 5.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.burns.2014.09.022.

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